MOLECULAR AND CELLULAR BIOLOGY, Jan. 1995, p. 365–378 Vol. 15, No. 1
0270-7306/95/$04.00+0

Structural Requirements for Double-Stranded RNA Binding, Dimerization, and Activation of the Human eIF-2α Kinase DAI in *Saccharomyces cerevisiae*

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Received 4 August 1994/Returned for modification 9 September 1994/Accepted 27 September 1994

The protein kinase DAI is activated upon viral infection of mammalian cells and inhibits protein synthesis by phosphorylation of the α subunit of translation initiation factor 2 (eIF-2α). DAI is activated in vitro by double-stranded RNAs (dsRNAs), and binding of dsRNA is dependent on two copies of a conserved sequence motif located N terminal to the kinase domain in DAI. High-level expression of DAI in *Saccharomyces cerevisiae* cells is lethal because of hyperphosphorylation of eIF-2α; at lower levels, DAI can functionally replace the protein kinase GCN2 and stimulate translation of *GCN4* mRNA. These two phenotypes were used to characterize structural requirements for DAI function in vivo, by examining the effects of amino acid substitutions at matching positions in the two dsRNA-binding motifs and of replacing one copy of the motif with the other. We found that both copies of the dsRNA-binding motif are required for high-level kinase function and that the N-terminal copy is more important than the C-terminal copy for activation of DAI in *S. cerevisiae*. On the basis of these findings, we conclude that the requirements for dsRNA binding in vitro and for activation of DAI kinase function in vivo closely coincide. Two mutant alleles containing deletions of the first or second binding motif functionally complemented when coexpressed in yeast cells, strongly suggesting that the active form of DAI is a dimer. In accord with this conclusion, overexpression of four catalytically inactive alleles containing different deletions in the protein kinase domain interfered with wild-type DAI produced in the same cells. Interestingly, three inactivating point mutations in the kinase domain were all recessive, suggesting that dominant interference involves the formation of defective heterodimers rather than sequestration of dsRNA activators by mutant enzymes. We suggest that large structural alterations in the kinase domain impair an interaction between the two protomers in a DAI dimer that is necessary for activation by dsRNA or for catalysis of eIF-2α phosphorylation.

A response to starvation and stress common to mammalian and yeast cells is the inhibition of protein synthesis by phosphorylation of the α subunit of translation initiation factor 2 (eIF-2α). The first step in translation initiation is the formation of a ternary complex composed of eIF-2 (made up of three nonidentical subunits), GTP, and charged initiator $tRNA^{Met}$ ($Met\text{-}tRNA_i^{Met}$). This ternary complex interacts with the small ribosomal subunit, forming a 43S preinitiation complex, which then binds to mRNA and assembles an 80S initiation complex at the AUG start codon. The eIF-2 is released from the ribosome in an eIF-2 · GDP binary complex. To initiate another round of translation, the GDP bound to eIF-2 must be replaced by GTP, which requires the guanine nucleotide exchange factor, eIF-2B (reviewed in reference 29). Phosphorylation of the α subunit of eIF-2 on the serine residue at position 51 impairs guanine nucleotide exchange on eIF-2 by inhibiting eIF-2B activity. As a consequence, the formation of new ternary complexes is diminished, and the rate of translation initiation decreases (reviewed in reference 13).

Three eIF-2α protein kinases have been identified in eukaryotic organisms: DAI (double-stranded RNA-activated inhibitor of translation) from humans and mice, HRI (heme-regulated inhibitor of translation) from rabbits, and GCN2 from the yeast *Saccharomyces cerevisiae*. GCN2 is activated when yeast cells are starved for amino acids or purines, and it specifically stimulates translation of *GCN4* mRNA, encoding a transcriptional activator of amino acid and purine biosynthetic genes (14). HRI is activated in reticulocytes in response to heme limitation and elicits a general inhibition of translation initiation (16). DAI is present in most mammalian cells and tissues and becomes activated in response to virus infection as a means of reducing total protein synthesis in the infected cells (26).

Purified or recombinant DAI can be activated in vitro by various natural or synthetic double-stranded RNAs (dsRNAs), and it is thought that dsRNA produced during the virus life cycle is responsible for DAI activation in infected cells (26). The N-terminal one-third of human DAI contains two regions rich in basic amino acids that show significant similarity with a sequence motif found in a number of unrelated dsRNA-binding proteins (38). The first copy of this sequence in DAI is a better match than the second to the dsRNA-binding motif. Deletions and clustered substitution mutations in the first copy of the motif severely impair dsRNA binding in vitro, whereas similar mutations in the second copy generally have lesser effects on dsRNA binding (2, 7, 11, 17, 27, 28, 34). The second copy of the motif can be functionally replaced by an additional copy of the first, whereas two copies of the second repeat cannot support dsRNA binding in vitro (11). Thus, while both

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copies of the motif are required for wild-type levels of dsRNA binding, the first makes a greater contribution than the second to in vitro binding activity.

Four lines of evidence indicate that the dsRNA-binding motifs in DAI mediate stimulation of DAI kinase activity by dsRNA in vivo. Expression of DAI in yeast cells lacking GCN2 leads to hyperphosphorylation of eIF-2α and a general inhibition of translation initiation and cell growth that is dependent on the presence of Ser-51 in eIF-2α (3, 5). Feng et al. (7) showed that four small deletions in the two dsRNA-binding motifs that reduced dsRNA binding in vitro also reduced the growth-inhibitory effect of expressing DAI in yeast cells. Two deletions in the first repeat impaired dsRNA binding more than two deletions in the second repeat and also more completely eliminated growth suppression by DAI in yeast cells. It was not ascertained, however, whether these deletions affected regulation of kinase activity, catalytic activity per se, or the stability of the enzyme in yeast cells. Second, mutation of Lys-64 in the first repeat of DAI impaired dsRNA binding in vitro and also decreased the ability of DAI to negatively regulate its own expression in transfected COS cells (27). DAI specifically inhibits its own translation in mammalian cells (2, 39), perhaps by localized phosphorylation of eIF-2 by newly synthesized enzyme present in the vicinity of its own mRNA. Third, several viral proteins that antagonize DAI function in infected cells contain the same dsRNA-binding motif found in DAI itself and have been shown to increase the concentration of dsRNA required to stimulate kinase activity in vitro (26). Finally, adenovirus produces large amounts of a highly structured RNA, known as adenovirus-associated (VA) RNA_I, that binds to DAI and prevents its activation in virally infected cells (26). The bindings of VA RNA_I and dsRNA activators to DAI have nearly identical structural requirements for the dsRNA-binding motifs (11), implying that binding of dsRNA activators to these sequences in DAI is an obligate step in kinase activation.

The way in which binding of dsRNA by DAI leads to kinase activation is not well understood. It is generally thought that dsRNA binding stimulates the autokinase activity of DAI and that autophosphorylation locks the kinase domain into a configuration that can bind and phosphorylate eIF-2α in the absence of dsRNA activators (8, 9, 20). The kinetics of activation are second order for enzyme concentration (20), and partially phosphorylated DAI purifies as a dimer, whereas unphosphorylated DAI exists as a monomer (21). These latter results could indicate that protein dimerization is required for activation of DAI kinase function. Optimal activation requires RNA duplexes greater than ca. 85 bp, and activation is inhibited by high concentrations of dsRNA less than ca. 30 bp in length (25, 33). If dimerization required that two molecules of DAI be bound to the same dsRNA molecule, this could explain why small dsRNAs and high concentrations of long dsRNAs both fail to activate DAI.

In this report, we analyze a large number of deletion and point mutations in the dsRNA-binding and catalytic domains of DAI for their effects on kinase activation and translational control of gene expression in *S. cerevisiae*. One of our goals was to investigate thoroughly whether reductions in dsRNA binding in vitro caused by mutations in the conserved sequence motifs lead to commensurate reductions in kinase activity in vivo. It was conceivable that the repeated motifs have a role in activating the catalytic domain in addition to binding dsRNA, in which case certain mutations would have greater effects on kinase function in vivo than on dsRNA binding in vitro. A second goal was to evaluate rigorously the relative contributions of the first and second copies of the dsRNA-binding motif to kinase activation in vivo. Toward this end, we analyzed substitutions of conserved amino acids located in equivalent positions of the two sequence motifs. The results of our analysis provide strong evidence that the level of kinase activity in yeast cells is proportional to the binding affinity of the N-terminal domain of the protein for dsRNA and that the first copy of the dsRNA-binding motif makes a greater contribution than the second to both dsRNA binding and kinase activation in vivo.

We also evaluated DAI mutants for the ability to interfere with the activation or function of the wild-type enzyme (dominant-negative phenotype). This inquiry was motivated by the observation that expression in mammalian cells of two different mutant DAI proteins with defects in the catalytic domain rendered cells tumorigenic, apparently by interfering with the function of the endogenous wild-type enzyme (19, 31). These findings led to the idea that DAI functions as a tumor suppressor in mammalian cells. Our results indicate that several deletions affecting the catalytic domain all have a dominant-negative phenotype in *S. cerevisiae*, whereas equally deleterious point mutations in kinase subdomains I and II are recessive. This unexpected finding leads us to suggest that the dominant-negative phenotype of catalytically inactive DAI proteins arises from formation of inactive heterodimers rather than from sequestration of dsRNA activators and that specific interactions between the two kinase domains in the heterodimer are required for kinase activation. Finally, we uncovered an instance of genetic complementation between different mutations in the dsRNA-binding domain, providing additional in vivo evidence that dimerization is required for the activation or catalytic function of DAI.

MATERIALS AND METHODS

Plasmids and yeast strains. Construction of the mutations in the N-terminal one-half of human DAI (11), the K296R point mutant (30), and the Δ6 deletion (19) were described previously. The cDNAs encoding the full-length DAI kinases were introduced into yeast cells on a derivative of the high-copy-number vector pEMBLyex4 containing a modified polylinker (11). This derivative of pEMBLyex4 was created by inserting a *Bam*HI site at the *Hin*dIII site of plasmid p1420 (5), followed by insertion of a *Hin*dIII site at the *Sst*I site of the resulting plasmid, destroying the original *Hin*dIII and *Sst*I sites in the process. All DAI cDNAs containing mutations in the N-terminal domain (except the Δ1 deletion), as well as the K296R mutation, were isolated as 1.8-kb *Hin*dIII-*Bam*HI fragments from pSRG2ΔL and related constructs described previously (11) and inserted between the *Hin*dIII and *Bam*HI sites of the modified pEMBLyex4 vector just described. The Δ1 mutation was constructed by inserting an *Nco*I-*Bam*HI fragment, in which the *Nco*I site was made blunt ended, between the *Hin*dIII and *Bam*HI sites of the modified pEMBLyex4 vector, after making the *Hin*dIII site blunt ended. Mutant DAI constructs 2:1 and 2:2, containing rearrangements in the dsRNA-binding domain, were created by transferring the *Xba*I-*Hin*dIII fragment containing the rearrangement from the appropriate pSRG2ΔL-derived construct (11) into the modified pEMBLyex4. A 520-bp *Bgl*II fragment containing the Δ6 mutation in the catalytic domain was isolated from plasmid p1643 (19) and inserted into *Bgl*II-digested p1469, which contains the wild-type DAI coding sequence on pEMBLyex4, creating plasmid p1671.

A PCR fusion technique (40) was used to construct the G274P and G279P point mutations, changing Gly-274 and Gly-279 to Pro by mutating their codons to CCC. The mutagenic primers used to generate the G274P mutation were complementary 45-mer oligonucleotides beginning at nucleotide 793 and ending at nucleotide 837, relative to the translation start site. The mutagenic primers used to make the G279P mutation were complementary 57-mer oligonucleotides beginning at nucleotide 793 and ending at nucleotide 849. The outside primers for these PCR fusions were 30-mer oligonucleotides beginning at nucleotides 654 and 1303, respectively. This strategy allowed the PCR fusion products to be inserted as *Bgl*II fragments into *Bgl*II-digested p1469, producing p1757 (G279P) and p1774 (G274P). p1543, used to construct the Δ7 mutation in catalytic subdomain II, contains the wild-type DAI allele on a *Hin*dIII-*Bam*HI fragment inserted into pUC18. PCR fusion was used to introduce *Hpa*I sites at V294 and N301 of DAI in p1543, using mutagenic complementary 45-mer oligonucleotide primers beginning at nucleotide 862 and ending at nucleotide 906. The outside primers were the same ones used to generate the G274P and G279P point mutations described above. The resulting PCR fusion product containing the two *Hpa*I sites was inserted into *Bgl*II-digested p1543, producing p1773. This con-

struct was digested with *Hpa*I and religated, generating p1765, which contains Δ7. A 520-bp *Bgl*II fragment containing the Δ7 deletion was isolated from p1765 and inserted into *Bgl*II-digested p1469, creating p1764. The ΔK mutant was created by deleting nucleotides 808 to 809, generating a stop codon at bp 861, and truncating the DAI protein at amino acid 271. p1549 was constructed to coexpress the DAI Δ2 mutant protein with other DAI alleles. A 2.8-kb *Apa*I-*Bam*HI fragment from p1472, which contains the DAI Δ2 allele in pEMBLyex4, was inserted into the high-copy-number *TRP1* yeast expression plasmid pRS424 (37) under the control of the yeast *GAL-CYC1* promoter. p1550 was constructed by inserting a 2.8-kb *Apa*I-*Bam*HI fragment from p1469, containing the wild-type DAI allele under control of the *GAL-CYC1* promoter, into the yeast integrating plasmid pRS305 (37).

The construction of yeast strain H1816 (**a** *ura3-52 leu2-3 leu2-112 trp1-Δ63 sui2Δ gcn2Δ* p1108[*GCN4-lacZ TRP1*] at *trp1-Δ63*, p1097[*SUI2 LEU2*]) has been described previously (6). Strain H1817 is isogenic to strain H1816 except that p1097[*SUI2 LEU2*] was replaced with p1098[*SUI2-S51A LEU2*]. Strain RY1-1 was created by digesting p1550 with *Eco*RV and transforming the linear fragment into strain H1894 (**a** *ura3-52 leu2-3 leu2-112 gcn2Δ trp1-Δ63*). DNA blot hybridization analysis confirmed that two copies of the wild-type DAI coding sequence, under control of the *GAL-CYC1* promoter, had integrated at the *LEU2* locus in strain RY1-1.

Immunoblot analysis of DAI protein expression. Transformants of strains H1816 and H1817 containing various DAI alleles were grown in synthetic minimal dextrose medium (SD) at 30°C for ~30 h and then shifted to inducing conditions, synthetic minimal medium containing 10% galactose and 2% raffinose (SGR), for ~12 h. Whole-cell extracts were prepared by breaking the cells with glass beads in lysis buffer (100 mM Tris-HCl [pH 8.0], 20% glycerol, 1 mM β-mercaptoethanol, 0.2 mM phenylmethanesulfonyl fluoride, 100 U of aprotinin per ml, 1 μg of pepstatin A per ml, 1 mM EDTA). Extracts were electrophoresed on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels and transferred to nitrocellulose. Blots were blocked in 1× TBS-T (20 mM Tris-HCl [pH 7.6], 150 mM NaCl, 0.1% Tween-20) with 5% nonfat dry milk. Immunodetection with DAI-specific polyclonal antiserum was done at a dilution of 1:3,500 in blocking solution. Subsequent steps and enhanced chemiluminescence detection were done as specified by the manufacturer (Amersham). The relative amounts of different mutant proteins expressed in strain H1817 were estimated by video image densitometry of the resulting autoradiograms, using the NIH Image 1.47 image processes and analysis software.

Production of polyclonal antiserum to DAI. The cDNA encoding the complete DAI protein was inserted into the pET11a vector (Novagen, Madison, Wis.) and used to transform *Escherichia coli* BL21(DE3)pLysS as previously described (1). After cultures had grown to an A_{550} of ca. 1.0, they were induced with isopropythiogalactopyranoside (IPTG) and grown for an additional 2 h. The cells were harvested and disrupted with lysis buffer (10 mM Tris-HCl [pH 7.5], 50 mM KCl, 1 mM dithiothreitol, 2 mM EDTA, 50 mM NaF, 0.5 mM phenylmethanesulfonyl fluoride, 1,000 U of aprotinin per ml, 1% Triton X-100). Following centrifugation, the supernatent fraction was diluted with buffer I (20 mM Tris-HCl [pH 7.5], 50 mM KCl, 400 mM NaCl, 1 mM EDTA, 100 U of aprotinin per ml, 1 mM dithiothreitol, phenylmethanesulfonyl fluoride, 0.5 mM, 20% glycerol, 1% Triton X-100) and incubated at 4°C for 2 h with anti-DAI monoclonal antibody bound to Sepharose 4B (22). The antibody-bound DAI was washed four times with buffer I and three times with buffer II (10 mM Tris-HCl [pH 7.5], 100 mM KCl, 0.1 mM EDTA, 100 U of aprotinin per ml, 20% glycerol). DAI was eluted by using guanidinium hydrochloride and dialyzed as previously described (9). For production of polyclonal antibody, New Zealand White rabbits were subcutaneously injected with 100 μg of the purified DAI expressed in *E. coli* along with an equal volume of Freund's complete adjuvant. Two successive injections of antigen mixed with 5 mg of poly(A) · poly(U) adjuvant were administered at 1- and 2-month intervals, respectively (15).

Analysis of *GCN4-lacZ* expression. β-Galactosidase activity in whole-cell extracts was assayed as previously described (24). After growth of strains exponentially for ~20 h, cultures were shifted to SGR medium for an additional 12 h prior to harvesting.

IEF PAGE of eIF-2α. Isoelectric focusing (IEF) polyacrylamide gel electrophoresis (PAGE) was conducted as previously described (6), with the following modifications. Yeast strains grown on plates containing synthetic minimal medium supplemented with 2% raffinose (SR) were inoculated into SR liquid medium and grown for ~18 h. Cultures were diluted 1:50 into SR medium containing 0.5% galactose, grown for 10 h, and harvested. Cells were broken with glass beads in IEF breaking buffer (6) containing 10 mM 2-aminopurine and 15 mM EDTA. Detection of eIF-2α by immunoblot analysis with antiserum prepared against a TrpE–eIF-2α fusion protein (4) was carried out by using enhanced chemiluminescence as described above.

In vitro protein kinase assays. Transformants of strain H1817 containing various DAI plasmid-borne alleles were grown for ~16 h in SD medium, diluted 1:50 into SGR medium, and grown for ~16 h. Cells were broken in kinase reaction lysis buffer (20 mM Tris-HCl [pH 8.0], 50 mM KCl, 400 mM NaCl, 20% glycerol, 1% Triton X-100, 0.5 mM EDTA). For immunoprecipitations, 150 μg of protein extract were incubated with DAI-specific antiserum for 2 h at 4°C. Immune complexes were collected as described by Wek et al. (39a), using protein A-Sepharose beads, washed three times with cold kinase reaction lysis buffer, and washed two times with cold 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.0). Phenylmethanesulfonyl fluoride was present in all solutions at 0.1 mM. The immune complexes were washed once with kinase reaction buffer (KRB) (20 mM HEPES [pH 7.0], 100 mM KCl, 2 mM $MnCl_2$, 2 mM $MgCl_2$, 0.5 mM dithrothreitol, 125 μM sodium orthovanadate), leaving a 40-μl suspension of beads after the final centrifugation. The kinase reaction was initiated by the addition of 10 μCi of [γ-^{32}P]ATP. The reaction mix was incubated at 30°C for 15 min, and the reaction was terminated by the addition of 40 μl of 2× SDS sample buffer. In reactions containing purified eIF-2, the substrate was preincubated at 50°C for 5 min and the kinase reactions were initiated after addition of 1 μg of the preincubated eIF-2. Samples were boiled for 10 min and analyzed by SDS-PAGE. Gels were stained with Coomassie blue, dried under vacuum, and subjected to autoradiography using Kodak XAR film. The eIF-2 was kindly provided by William C. Merrick (Case Western Reserve University, Cleveland, Ohio).

RESULTS

Genetic measurements of in vivo kinase activity for mutant DAI proteins expressed in *S. cerevisiae*. The N-terminal half of human DAI contains three regions rich in basic amino acids, of which the first two are homologous to the dsRNA-binding motif. Green and Mathews (11) previously described deletions and rearrangements that either removed each of these basic regions, duplicated the first or second copies of the dsRNA-binding motif, or switched the order of these two repeats (Fig. 1A). Additionally, linker-scanner mutations that changed basic amino acids to nonpolar residues were made in the first or second copy of the dsRNA-binding motif. More recently, three pairs of single amino acid changes were made to replace conserved residues at equivalent positions in the two repeats (Fig. 1B) (10). Mutations LS9, LS16, and LS19 were designed specifically to examine the importance of secondary structure in each repeat by replacing a conserved alanine residue with proline in the predicted α-helical region in the C-terminal portion of each motif.

The N-terminal segments of all the DAI mutant alleles were assayed previously for the ability to bind dsRNA in vitro, and the results of these measurements are summarized in Table 1. These data showed that the third basic region in DAI was dispensable, whereas deletion of either copy of the dsRNA-binding motif essentially abolished dsRNA binding in vitro (Δ1, Δ2, and Δ3 in Fig. 1A and Table 1) (11). Analysis of point mutations at equivalent positions in the two copies of the binding motif indicated that the first repeat was more important than the second for dsRNA binding in vitro (e.g., compare LS17 with LS14, LS18 with LS15, and LS19 with LS16 in Fig. 1B and Table 1). In accord with this conclusion, it was found that the second repeat could be replaced with an additional copy of the first repeat without a substantial loss in dsRNA binding but that the converse was not true (1:1 and 2:2 alleles in Fig. 1A and Table 1). In addition, switching the order of the two repeats actually led to a significant increase in the efficiency of dsRNA binding (11, 10).

We analyzed the effects of these mutations on DAI kinase function in vivo by expressing the mutant proteins in yeast strains lacking the endogenous eIF-2α kinase GCN2. Full-length cDNAs encoding each of the mutant DAI alleles were inserted into the yeast expression vector pEMBLyex4 and produced in yeast cells under the control of the galactose-inducible *GAL1-CYC1* promoter. We and others showed previously that high-level expression of wild-type DAI from this promoter on galactose medium inhibits yeast cell growth as a result hyperphosphorylation of eIF-2α on Ser-51 (3, 5). Accordingly, this growth inhibition is completely eliminated either by a mutation in DAI that inactivates the kinase domain (substitution of Lys-296 in kinase subdomain II with arginine [K296R]) or by substitution of Ser-51 on eIF-2α with alanine (eIF-2α-S51A).

In cells grown on medium containing glucose as the carbon

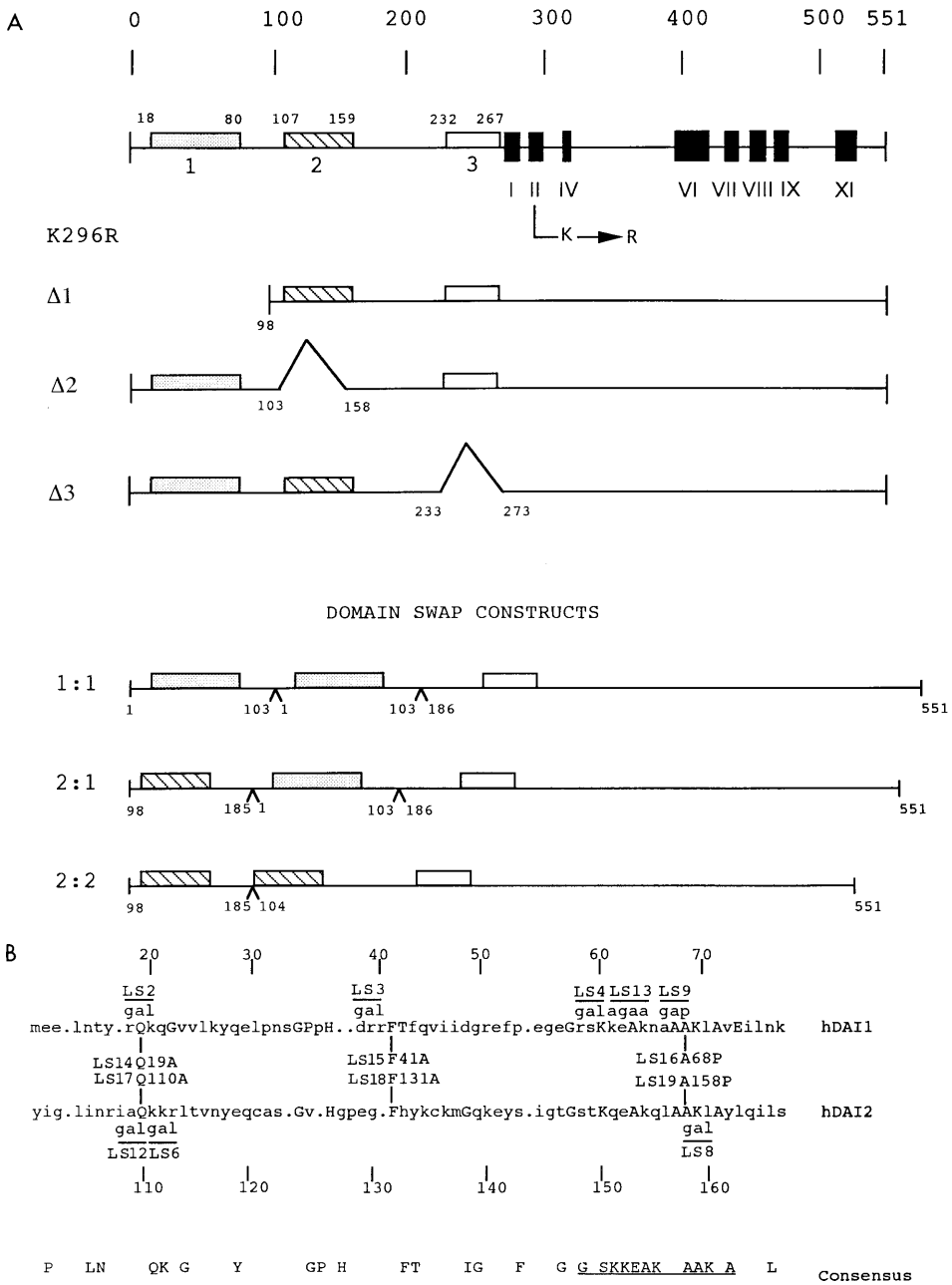

FIG. 1. Structures of DAI mutants. (A) Schematic representation of wild-type and mutant DAI proteins. The top line indicates the full-length wild-type DAI protein sequence. The boxes in the amino-terminal half of the diagram represent the three regions rich in basic residues. The amino acid residues spanning these regions are indicated. The black boxes in the C-terminal one-half of the protein (I to XI) represent conserved kinase subdomains found in the catalytic regions of all protein kinases. The K296R inactivating point mutation in kinase subdomain II is indicated. The lower diagrams show the mutants containing deletions of the amino-terminal basic regions and also mutants containing rearrangements of dsRNA-binding domains 1 and 2. The numbers correspond to amino acid residues in the wild-type protein. (B) Locations of linker-scanner mutations and point mutations made in the two dsRNA-binding domains. Amino acid residues 11 to 77 (hDAI1) and 101 to 167 (hDAI2), containing the two dsRNA-binding motifs in human DAI, are shown. A dsRNA-binding consensus sequence motif, derived from characterized dsRNA-binding proteins, is shown on the last line (11). Highly conserved amino acid residues within hDAI1 and hDAI2 are indicated in uppercase letters. The most conserved C-terminal portion of the motif, predicted to form an α-helical region, is underlined in the consensus sequence. The names of mutations created by linker-scanning site-directed mutagenesis are underlined and show the mutant residues directly above the wild-type residues that they replaced. Point mutations of equivalent conserved amino acids in the two domains, the positions of the mutations, and the amino acid changes are also indicated (LS14 to LS19).

source, the *GAL1-CYC1* promoter is repressed and a very low level of DAI is produced; this level of DAI has no effect on cell growth but is sufficient to restore translation of *GCN4* mRNA in strains lacking GCN2, provided that eIF-2α contains a serine residue at position 51. Yeast strains lacking GCN2 or expressing nonphosphorylatable eIF-2α-S51A fail to grow on media containing 3-aminotriazole (3-AT), a competitive inhibitor of histidine biosynthesis, because they cannot derepress *GCN4* and its target genes in the histidine biosynthetic pathway. Thus, suppression of the 3-AT-sensitive phenotype of a *gcn2* strain can be used as a sensitive indicator of DAI kinase activity in vivo (5). In addition, the translational derepression

TABLE 1. In vitro dsRNA-binding and kinase function for mutant DAI alleles expressed in *gcn2*Δ yeast strains

Kinase activity	DAI allele[a]	Location of mutation[b]	dsRNA binding[c] (%)	Growth[d]			Relative *GCN4-lacZ* expression[e]	Relative protein levels in H1817[f]
				SGAL	SGAL + 3-AT	SD + 3-AT		
Hyperactivated	2:1	Domain swap	130	−−	−−	+++	5[g]	1.6
WT[h]	WT	WT	100	−−	−−	++	6	1.0
	LS8	2/158-160	116	−−	−−	++	5	0.7
	LS18[1]	2/F131A	90	−−	−−	++	8	0.9
	LS3	1/38-40	85	−−	−−	++	7	0.9
	1:1	Domain swap	80	−−	−−	+++	7	2.0
	LS6	2/111-113	10–15	−−	−−	++	4	1.3
Reduced								
I	LS17[2]	2/Q110A	~50	−−	−−	−/+	4	1.0
	LS14[2]	1/Q19A	~20	−−	−−	−/+	7	1.0
	LS12	2/108-110	10	−−	−−	−/+	4	0.7
II	LS15[1]	1/F41A	10	−−	−−	−−	3	0.8
	LS19[3]	2/A158P	~5	−−	−−	−−	3	0.7
	LS2	1/18-20	0–5	−−	−−	−−	2	1.3
	LS4	1/58-60	0–5	−−	−−	−−	3	1.0
	LS13	1/61-64	0–5	−−	−−	−−	2	0.8
Low								
I	Δ2	2/Δ103-158	0–5	−/+	+	−−	2	0.5
II	LS9	1/66-68	0–5	+	+	−−	1	0.3
III	Δ1	1/Δ1-98	0–5	+	−/+	−−	1	0.3
	LS16[3]	1/A68P	0–5	+	−/+	−−	1	0.3
No activity	2:2	Domain swap	0–5	++	−−	−−	1	0.7
	K296R	296	100	++	−−	−−	1	1.0
	Δ3	3/Δ233-273	100	++	−−	−−	1	1.0

[a] DAI alleles containing equivalent point mutations in dsRNA-binding domains 1 and 2 are indicated with the same superscript (1, 2, or 3).
[b] dsRNA-binding domain in which the mutation was made/residue(s) changed, as indicated in Fig. 1.
[c] In vitro dsRNA binding by the N-terminal segments of DAI mutant alleles, expressed as a percentage of wild-type binding. These data were obtained by Green et al. (10, 11).
[d] Transformants of strain H1816 bearing the plasmid-borne DAI constructs listed on the left were analyzed for growth on the indicated media as described in the legend to Fig. 2. Growth was scored qualitatively relative to that of wild-type DAI transformants.
[e] Transformants of strain H1816 were grown for 12 h under inducing conditions (SGR medium containing 10% galactose and 2% raffinose), and β-galactosidase activities, expressed from an integrated wild-type *GCN4-lacZ* fusion, were measured in whole-cell extracts. Enzyme activities, averaged from two or more independent transformants, are expressed relative to that given by vector alone (assigned a value of 1).
[f] Transformants of strain H1817 expressing mutant eIF-2α-S51A were grown under inducing conditions, and the levels of DAI protein were measured in whole-cell extracts by immunoblot analysis. The amounts of each mutant protein measured in three or more immunoblots were averaged and expressed relative to that determined for wild-type DAI.
[g] Relative β-galactosidase activity measured after ~20 h of exponential growth under noninducing conditions (glucose-containing SD medium).
[h] WT, wild type.

of *GCN4* mediated by DAI can be measured quantitatively by assaying β-galactosidase expression from a *GCN4-lacZ* fusion. Finally, because DAI negatively autoregulates its own expression in yeast cells (5), the level of DAI kinase activity can be assessed by measuring the steady-state level of DAI protein. By determining DAI protein levels in isogenic strains containing either wild-type eIF-2α or eIF-2α-S51A, we could distinguish between effects on DAI expression due to autoregulation (seen in the presence of wild-type eIF-2α) from differences in protein stability or immunological cross-reactivity (seen in the presence of eIF-2α-S51A).

The mutant DAI alleles listed in Table 1 were introduced on expression plasmids into isogenic yeast strains H1816 and H1817, expressing wild-type eIF-2α and eIF-2α-S51A, respectively. The resulting transformants were tested for their effects on growth in medium containing galactose (SGAL) or dextrose (SD) as the carbon source and in the presence or absence of 3-AT. The different DAI mutant alleles could be assigned to one of eight distinguishable categories with respect to growth on these different media. One group of five alleles (LS8, LS18, LS3, 1:1, and LS6) gave a pattern of growth in H1816 that was very similar to that of the wild-type DAI construct. High-level expression of these alleles on galactose medium was lethal, whereas low-level expression on dextrose medium led to induction of *GCN4* and resistance to 3-AT but had no effect on growth in medium lacking 3-AT (Table 1; 1:1 in Fig. 2A). As judged from its growth phenotype on SD plus 3-AT, the 1:1 construct seems to confer a level of kinase activity somewhat higher than that conferred by wild-type DAI (Table 1). In strain H1817, containing eIF-2α-S51A, expression of these five alleles (and all other DAI constructs listed in Table 1) had no effect on growth in any of the four media tested. The five constructs with growth phenotypes similar to that of wild-type DAI included the four mutant alleles with the highest levels of dsRNA binding in vitro plus LS6, which showed reduced dsRNA binding (Table 1).

The 2:1 construct exhibited increased dsRNA binding in vitro and was the only allele with growth phenotypes indicating a level of kinase function substantially in excess of that of wild-type DAI. First, H1816 transformants bearing this construct formed colonies more slowly than those with wild-type DAI even under noninducing conditions on SD medium. This slow-growth phenotype was not observed in H1817, showing that it arose from hyperphosphorylation of eIF-2α and inhibi-

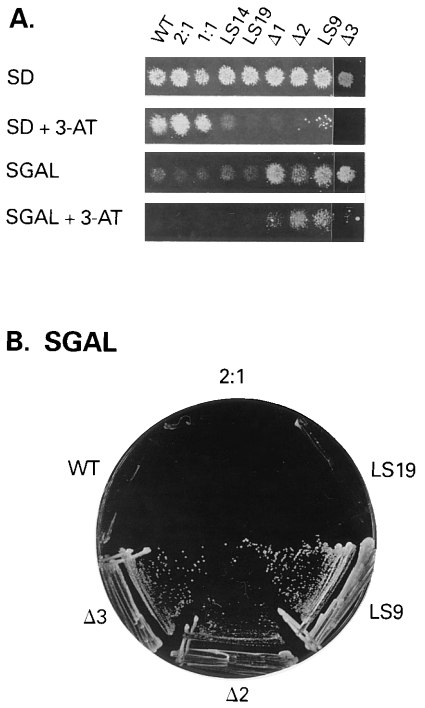

FIG. 2. Amino acid analog sensitivity and growth rate analysis of yeast strains expressing wild-type and mutant DAI proteins. (A) Plasmids carrying the indicated DAI alleles were introduced into the *gcn2*Δ strain H1816 expressing wild-type (WT) eIF-2α. Patches of transformants were grown to confluence on minimal SD medium and replica printed to SD, SD plus 30 mM 3-AT, SGAL medium (10% galactose, 2% raffinose), and SGAL plus 30 mM 3-AT. Plates were incubated for 3 to 4 days at 30°C. (B) Transformants of H1816 strains expressing the indicated DAI proteins with different kinase activities were streaked for single colonies on SGAL medium and incubated for 5 to 6 days at 30°C.

tion of general protein synthesis in H1816. The 2:1 construct also conferred greater resistance than did wild-type DAI to 3-AT in SD medium (Table 1; Fig. 2A), indicating more efficient induction of *GCN4* translation under these conditions. As discussed below, this interpretation was borne out by measurements of *GCN4-lacZ* expression.

Eight DAI mutant alleles containing point mutations in the first or second copy of the repeat had growth phenotypes indicative of reduced kinase activity in vivo (Table 1, LS17, LS14, LS12, LS15, LS19, LS2, LS4, and LS13). These constructs were lethal when induced on galactose media (Fig. 2A, LS14 and LS19, and Fig. 2B, LS19); however, they conferred either a reduced level of 3-AT resistance (subgroup I) or 3-AT sensitivity in SD medium (subgroup II) (Table 1; Fig. 2A, LS14 and LS19). All of the constructs in this group exhibited either reduced or negligible dsRNA-binding activity in vitro (Table 1). Note that the members of subgroup II, which have less kinase function than those in subgroup I (judging by their greater sensitivity to 3-AT), also were generally more impaired for dsRNA binding in vitro than were constructs in subgroup I (Table 1).

Four of the mutants (Δ2, LS9, Δ1, and LS16) had phenotypes indicative of very low levels of kinase function in vivo (Table 1). The alleles in this group contain either complete deletions of one of the two repeats or a proline substitution in the putative α-helical segment of the first repeat, and all four showed negligible dsRNA binding in vitro. Each of these constructs conferred slow growth rather than lethality on SGAL medium (Fig. 2B, Δ2 and LS9), indicating that general translation was only partially impaired when DAI expression was highly induced on galactose medium. The Δ2 construct appeared to retain the most kinase activity in this group, being significantly more growth suppressive on SGAL medium than the other three alleles. As expected, none of the four alleles in this group conferred resistance to 3-AT in SD medium but all did so when expressed at high levels in SGAL medium (Table 1; Fig. 2A, Δ1, Δ2, and LS9). On the basis of this phenotype, Δ2 and LS9 appeared to have kinase activities somewhat greater than those of Δ1 and LS16 (Fig. 2A and data not shown). Thus, these four alleles exhibited three distinguishable levels of kinase activity (Δ2 > LS9 > Δ1, LS16; Table 1).

The final group of three constructs had growth phenotypes indicative of no kinase activity in vivo. These include two mutations in the N-terminal half of DAI (the 2:2 domain swap and Δ3) that exhibit, respectively, negligible and wild-type levels of dsRNA binding in vitro. This group also contains the K296R mutation in kinase subdomain II that abolishes kinase catalytic activity (30). Strains carrying these nonfunctional alleles grew on the various test media indistinguishably from strains transformed with the vector alone, i.e., robust growth on SD and SGAL and no growth on SD plus 3-AT and SGAL plus 3-AT (Table 1; Fig. 2, Δ3).

To confirm our interpretation of the relationship between growth phenotypes and level of DAI kinase activity in vivo, we analyzed a *GCN4-lacZ* fusion whose expression is coupled translationally to the level of eIF-2α phosphorylation in the cell. Induction of the wild-type DAI construct in strain H1816 led to a ca. 6.0-fold increase in *GCN4-lacZ* expression relative to that seen in transformants containing vector alone (Table 1). For the DAI mutants judged by growth tests to have essentially wild-type levels of kinase activity, *GCN4-lacZ* expression was between four- and eightfold higher than that seen with vector alone, comparable to the sixfold induction conferred by wild-type DAI (Table 1). Interestingly, the hyperactivated 2:1 domain swap mutant showed elevated *GCN4-lacZ* expression under noninducing conditions. This result is in accord with the hyperresistance to 3-AT seen under noninducing conditions for this allele (Table 1). Under inducing conditions, *GCN4-lacZ* expression in the transformants bearing the 2:1 construct was not significantly higher than that given by vector alone. We attribute the latter observation to severe inhibition of protein synthesis when the 2:1 construct is induced on galactose medium.

In general, the DAI mutants assigned to the class with reduced kinase activity stimulated *GCN4-lacZ* expression less than was seen for kinases in the wild-type category. Moreover, higher levels of *GCN4-lacZ* expression were observed for the three kinases assigned to subgroup I of the reduced-activity class than for the five alleles in subgroup II of this category (Table 1). In the low-activity class of kinases, only Δ2 gave a significant induction of *GCN4-lacZ* expression (twofold), and this construct was judged to be the most functional member of this category on the basis of its slow-growth phenotype on SGAL (Table 1). Finally, the three alleles judged to have no kinase activity also showed no induction of *GCN4-lacZ* expression. We conclude that the magnitude of *GCN4-lacZ* expression conferred by the different DAI constructs is in good agreement with the levels of kinase activity assigned to these alleles on the basis of growth phenotypes.

Effects of DAI mutations on the autoregulation of DAI expression in *S. cerevisiae*. DAI efficiently down-regulates its own translation in mammalian cells (2). In yeast cells, catalytically inactive DAI is expressed at higher levels than wild-type DAI, and wild-type DAI is produced at higher levels in yeast strains

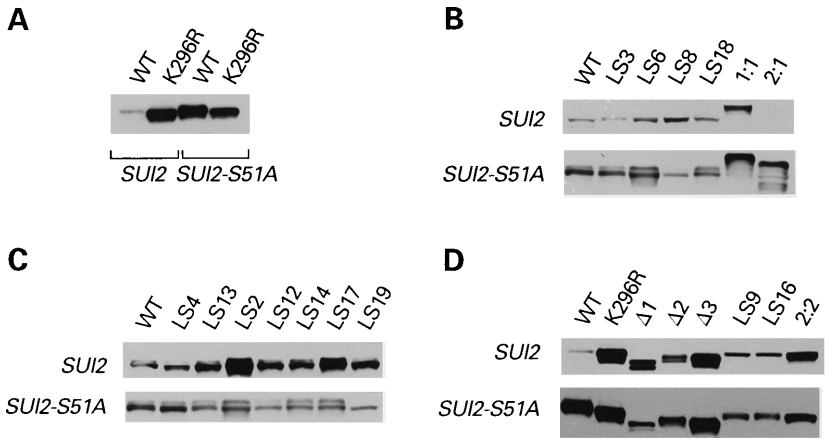

FIG. 3. Immunoblot analysis of induced DAI protein levels in *gcn2*Δ yeast strains expressing eIF-2α or eIF-2α-S51A. (A) Strains expressing wild-type (WT) DAI or the K296R inactive kinase were grown under inducing conditions (in SGR medium) for ~12 h, and whole-cell extracts were prepared. Fifteen micrograms of total cell protein from yeast strains H1816 (*SUI2*) and H1817 (*SUI2-S51A*) expressing wild-type DAI or the K296R mutant was subjected to SDS-PAGE and immunoblot analysis using DAI-specific polyclonal antiserum and an enhanced chemiluminescence system to detect immune complexes. (B) Protein expression by DAI alleles classified as having wild-type kinase activity. Thirty and 15 μg of total cell protein were analyzed, as described for panel A, for the H1816 and H1817 transformants, respectively. (C) Protein expression by DAI alleles in the reduced-activity class. Twenty and 15 μg were analyzed for the H1816 and H1817 transformants, respectively. (D) Protein expression levels from nonfunctional and low-activity DAI alleles. Fifteen micrograms of total cell protein was analyzed for transformants of both H1816 and H1817.

containing eIF-2α-S51A than in strains containing wild-type eIF-2α (5). These findings strongly suggest that DAI negatively regulates its own translation in yeast cells as in mammalian cells. According to this hypothesis, DAI protein levels should be inversely proportional to the in vivo kinase activity of mutant DAI alleles. To test this idea, we used immunoblot analysis to measure the levels of mutant DAI proteins after growing yeast cells in galactose medium. Extracts were prepared from transformants of H1816 (expressing wild-type eIF-2α), in which DAI expression should be autoregulated, and from H1817 (expressing eIF-2α-S51A), in which no autoregulation should occur. We reasoned that protein levels would be similar for all of the DAI alleles in strain H1817 unless there were differences in mRNA or protein stability for different constructs or if certain mutant proteins reacted differently with the antiserum.

We estimated that expression of wild-type DAI was ~50-fold lower than that of the nonfunctional K296R mutant in strain H1816, expressing wild-type eIF-2α, whereas the two kinases were expressed at similar levels in strain H1817, expressing eIF-2α-S51A (Fig. 3A). Among the five DAI alleles that exhibited essentially wild-type activity as judged by growth phenotype and *GCN4-lacZ* expression, LS3, LS6, and LS18 had protein levels very similar to that of wild-type DAI in both H1816 and H1817 (Fig. 3B). The 1:1 construct was present at levels about twofold higher than the wild-type level, but this occurred in both strains (Fig. 3B). Thus, either DAI protein or mRNA from the 1:1 construct is more stable than that of wild-type DAI or the 1:1 protein reacts better with the antiserum than does the wild-type protein. LS8 differed from the other constructs in this class by being expressed, on average, 1.5-fold higher than wild-type DAI in strain H1816 but at only 70% of the wild-type level in H1817 (Fig. 3B; the protein level shown for LS8 in the *SUI2-S51A* strain is lower than was seen in other replicate determinations). This finding could indicate that the LS8 protein has somewhat lower kinase activity than the other members of this group, a possibility consistent with the slightly lower *GCN4-lacZ* expression conferred by this construct compared with wild-type DAI (Table 1). Only very low levels of the 2:1 domain swap protein were detected in the H1816 strain even though it was expressed at roughly wild-type levels in the H1817 strain (Fig. 2B). This last result supports the idea that the 2:1 construct encodes a hyperactivated kinase that negatively regulates its own synthesis to a greater extent than does wild-type DAI.

The mutant DAI proteins assigned to the group with reduced kinase activity were expressed at two- to four-times-higher levels than the wild type in the H1816 strain but at levels similar to the wild-type level (or ca. 50% of the wild-type level in the case of LS12 and LS19) in strain H1817 (Fig. 3C). Thus, the enzymes in the reduced-activity class generally showed decreased negative autoregulation of DAI expression. The proteins in the low-activity group accumulated to even higher levels relative to wild-type DAI (three- to fivefold) in strain H1816, despite the fact that their levels appeared to be only 30 to 50% of the wild-type level in strain H1817 (Δ1, Δ2, LS9, and LS16 in Fig. 3D). The mutants assigned to the class of inactive kinases all accumulated to the very high levels characteristic of the K296R mutant (Fig. 3D, K296R, Δ3, and 2:2). We conclude that the relative amounts of DAI proteins expressed in the strain containing wild-type eIF-2α are in general accord with the levels of in vivo kinase activity that were assigned to different mutants on the basis of growth phenotypes and *GCN4-lacZ* expression.

The DAI protein levels in strain H1817, containing eIF-2α-S51A (shown in Fig. 3), were quantitated and listed in Table 1. These data indicate that most of the mutant proteins were expressed in H1817 at levels very similar to that of wild-type DAI. The greatest differences (two- to threefold less than the wild-type level) were observed for the low-activity class of enzymes. It is unlikely that reductions in protein stability of this magnitude could account for the low-level kinase function observed for these proteins, because they accumulated to higher levels than did enzymes assigned to the higher-activity classes when examined in strain H1816, containing wild-type eIF-2α (Fig. 3). Thus, the effects of negative autoregulation on protein accumulation in strain H1816 greatly outweighed any possible differences in stability among the mutant proteins.

Biochemical measurements of eIF-2α phosphorylation in vivo by wild-type and mutant DAI kinases. The results pre-

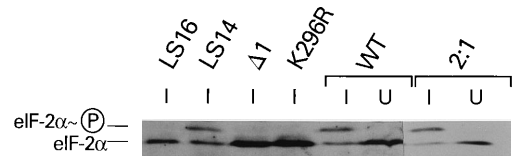

FIG. 4. IEF PAGE of eIF-2α from *gcn2*Δ strains expressing DAI alleles with various kinase activities. eIF-2α phosphorylation was measured in transformants of strain H1816 expressing the indicated DAI alleles. Strains were grown under inducing (I) conditions (SGR medium), in which the DAI protein is highly expressed, or in uninducing (U) conditions (glucose containing SD medium), in which DAI expression is minimal. Samples of total cell proteins were separated by IEF on a vertical slab gel as described previously (5) and subjected to immunoblot analysis with polyclonal antiserum specific for eIF-2α. Immune complexes were detected by using an enhanced chemiluminescence system. The positions of the basally phosphorylated form of eIF-2α and of eIF-2α phosphorylated on serine residue 51 are indicated. WT, wild type.

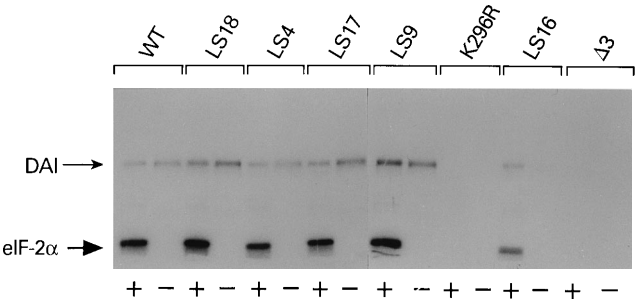

FIG. 5. In vitro kinase activities of immunopurified DAI proteins expressed in *gcn2*Δ yeast strains. Transformants of the H1817 strain containing the indicated DAI alleles were grown under inducing conditions, and DAI proteins were immunoprecipitated from aliquots of whole-cell extracts containing 150 μg of total protein. Immune complexes were incubated in kinase reaction buffer in the presence of [γ-^{32}P]ATP, with (+) or without (−) the addition of 1 μg of purified rabbit eIF-2, for 15 min at 30°C. Radiolabeled samples were analyzed by SDS–PAGE (10% polyacrylamide gel) followed by autoradiography. WT, wild type.

sented thus far indicated that mutations in the dsRNA-binding motifs of DAI reduce its ability to stimulate *GCN4* expression, to negatively regulate its own expression, and to impair general protein synthesis. Consequently, we expected that these mutations would reduce phosphorylation of eIF-2α in vivo. To confirm this expectation, we measured the level of eIF-2α phosphorylation in H1816 transformants bearing representative alleles from each activity class following induction of the enzymes on galactose medium. IEF PAGE was used to resolve and quantitate the proportion of eIF-2α that is phosphorylated on Ser-51.

As in previous studies (6), all of the eIF-2α present in untransformed H1816 resolved as a single band on IEF polyacrylamide gels at the position corresponding to eIF-2α that is not phosphorylated on Ser-51, because this strain lacks GCN2 (data not shown). When wild-type DAI was induced in H1816 transformants by growth on galactose medium, eIF-2α phosphorylated on Ser-51 was present in excess of the nonphosphorylated form (Fig. 4) (5). As expected, induction of the catalytically defective K296R kinase gave rise to no phosphorylation on Ser-51. The latter result was also obtained for the LS16, Δ1, and Δ2 mutant kinases from the low-activity class (Fig. 4, LS16, Δ1, and data not shown). In contrast, induction of the LS14 protein from the reduced-activity class led to phosphorylation of less than 50% of the eIF-2α. Finally, induction of the hyperactivated 2:1 enzyme led to a higher proportion of eIF-2α phosphorylated on Ser-51 than occurred with wild-type DAI (Fig. 4). (Note that the hyperactivated 2:1 kinase did not phosphorylate Ser-51 under noninducing conditions because cells were harvested in mid-exponential growth phase when little or no kinase is being expressed from the *GAL1-CYC1* promoter [5].) These results are in accord with the relative activities of the mutant and wild-type kinases deduced from the phenotypes shown in Table 1 and Fig. 3.

In vitro kinase activities of wild-type and mutant DAI proteins immunoprecipitated from *S. cerevisiae*. It was conceivable that mutations in the dsRNA-binding motifs reduced DAI function in vivo by causing a structural change in the kinase domain that destroyed catalytic activity, rather than by impairing stimulation of kinase activity by dsRNA. In an effort to eliminate this possibility, we conducted in vitro kinase assays on several mutant DAI alleles representative of different activity classes shown in Table 1. DAI proteins were induced in yeast cells by growth on SGAL medium and immunoprecipitated from whole-cell extracts. In accord with previous findings (3), DAI immunopurified from yeast extracts exhibited kinase activity without the addition of dsRNA activators, and addition of poly(I-C) at 0.01 to 0.1 μg/ml produced no significant increase in activity for either mutant or wild-type enzymes. We found, unexpectedly, that immunopurified DAI proteins from the reduced-activity (LS4 and LS17) and low-activity (LS9 and LS16) groups showed autophosphorylation and phosphorylation of the α subunit of purified eIF-2 at levels similar to that seen for the wild-type enzyme (Fig. 5). The same was true at time points in the reactions earlier than those shown in Fig. 5. Note that the different kinases were immunoprecipitated from transformants of strain H1817 in which wild-type and mutant proteins were present at similar levels (Fig. 3B and D). From these findings, we conclude that the in vitro-specific activities of the mutant kinases encoded by the LS18, LS4, LS17, LS9, and LS16 constructs are within 50% of the specific activity of wild-type DAI. Similar results were obtained for the mutant kinases encoded by LS6 and Δ1 (data not shown). The only DAI mutants that were nonfunctional in the in vitro assays were K296R and Δ3 (Fig. 5). These findings indicate that the reduced in vivo kinase function observed for DAI alleles containing mutations in the repeated dsRNA-binding motifs results not from a defect in catalytic function per se but from a defect in the activation of the kinases. Presumably, the concentration of dsRNA activators in the immunopurified kinase preparations is high enough to compensate for the reduced dsRNA binding caused by mutations in the repeated motifs. Alternatively, binding of DAI to antibodies may stimulate its kinase activity independently of dsRNA. The former explanation is favored by the fact that the immunopurified enzymes were inhibited at poly(I-C) concentrations of 0.05 and 0.1 μg/ml (data not shown).

Identification of dominant-negative DAI mutations. Previous studies have shown that mutant DAI proteins can interfere with the function of the wild-type enzyme when the two proteins are coexpressed in the same cells. For example, expression of a mutant kinase containing a deletion of residues 361 to 366 in kinase subdomain V (known as Δ6) in COS-1 cells led to a ca. 60% reduction in autophosphorylation by the wild-type enzyme being expressed in the same cells (19). In addition, the K296R mutant reversed the inhibition of translation in reticulocyte lysates by dsRNA, presumably by sequestering dsRNA and interfering with activation of the endogenous wild-type kinase (36). In yeast cells, it was shown that expression of a truncated DAI protein containing only the dsRNA-binding motifs in the N terminus reversed the growth inhibition elicited by wild-type DAI, and again it was proposed that the mutant protein prevented activation of wild-type DAI by sequestering

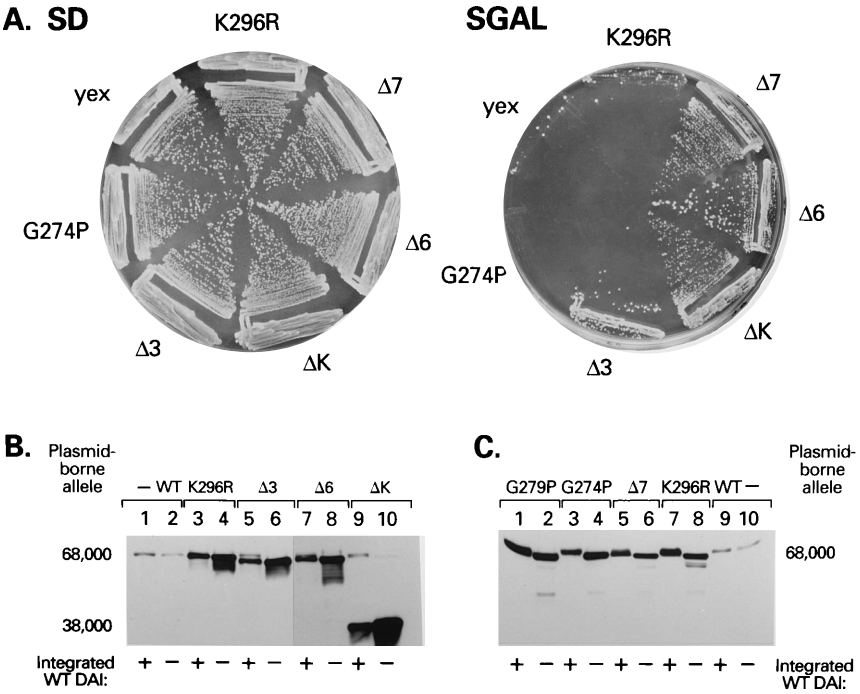

FIG. 6. Growth rates and immunoblot analysis of yeast strains coexpressing wild-type and mutant DAI protein kinases. (A) Plasmids carrying the indicated DAI alleles or vector alone (yex) were introduced into strain RY1-1, which contains two copies of the wild-type DAI coding sequence integrated at the *LEU2* locus. Strains were streaked on SD and SGAL (10% galactose, 2% raffinose) plates and incubated at 30°C for 3 to 4 and 5 to 7 days, respectively. (B) Immunoblot analysis of DAI protein levels in strains H1816 and RY1-1. Transformants bearing the indicated wild-type (WT) or mutant DAI alleles in strain RY1-1 (lanes 1, 3, 5, 7, and 9) or H1816 (lanes 2, 4, 6, 8, and 10) were grown under inducing conditions, and 15 μg of total cell protein was subjected to immunoblot analysis as described for Fig. 3. The − above lane 1 indicates that this RY1-1 transformant carried the empty vector. Two molecular weight markers are indicated at the left. (C) Immunoblot analysis of DAI proteins conducted as described for panel B showing expression of the Δ7, G274P, and G279P proteins in strains RY1-1 and H1816.

dsRNA activators (3). We reasoned that if this explanation was correct, mutations in the dsRNA-binding motifs that inactivate DAI in *S. cerevisiae* should not have a dominant-negative effect on the wild-type enzyme.

To test this prediction, we constructed strain RY1-1, which contains two copies of the wild-type DAI construct integrated into the chromosome at the *LEU2* locus as well as a deletion of *GCN2*. As expected, this strain is 3-AT resistant on SD medium and fails to grow when DAI is expressed to high levels on SGAL medium (Fig. 6A, sector labeled yex). We examined whether any of several mutant DAI alleles listed in Table 1 had a dominant-negative effect when produced in strain RY-1 from the same expression plasmids used earlier. We also examined the Δ6 mutant lacking residues 361 to 366 described above and a mutant truncated at residue 271 that contains both dsRNA-binding motifs but completely lacks the protein kinase domain (ΔK). As expected, both of these deletion mutants were catalytically inactive in immune complex assays of the type shown in Fig. 5 (data not shown). We found that Δ6, ΔK, and the catalytically inactive Δ3 construct restored growth of strain RY1-1 on galactose medium (Fig. 6A) but did not reverse the 3-AT resistance phenotype of RY1-1 in SD medium (data not shown). These results indicate that these three deletion alleles are dominant-negative constructs that do not completely inactivate the wild-type enzyme. In contrast, alleles with wild-type kinase domains and defective dsRNA-binding domains, including LS9, LS14, LS15, LS16, Δ1, and Δ2, showed no dominance over the wild-type allele for either phenotype when expressed in the RY1-1 strain (data not shown).

These were the results expected if the dominant-negative phenotype arose from sequestration of dsRNA activators by mutant enzymes, as Δ3, Δ6, and ΔK all contain intact dsRNA-binding domains. Inconsistent with this mechanism, however, we found that expressing the catalytically inactive K296R allele in strain RY1-1 did not interfere with wild-type DAI and restore growth on SGAL (Fig. 6A, K296). Because the K296R allele contains a wild-type dsRNA-binding domain, it would be expected to sequester dsRNA activators and interfere with activation of wild-type DAI to the same extent seen for the Δ3, Δ6, and ΔK enzymes. In an effort to define better the structural requirements for the dominant-negative phenotype, we constructed additional alleles that were catalytically inactive as a result of mutations in the protein kinase domain. Two different point mutations in kinase subdomain I (G274P and G279P) and a six-residue deletion of residues Ile-295 to Tyr-300 that encompasses invariant Lys-296 (Δ7) were all found to be catalytically inactive in immune complex assays (data not shown). When expressed in strain RY1-1, however, only Δ7 had a dominant-negative phenotype and restored growth on SGAL (Fig. 6A, G274P and Δ7). Thus, among the seven catalytically inactive constructs that we examined, the four deletion alleles showed dominance and the three point mutations were recessive. After repeated examination of the four dominant-negative alleles for their effects on growth of strain RY1-1 on SGAL and SD plus 3-AT, we found that Δ6 and Δ3 showed greater dominance, whereas ΔK showed less dominance, than was seen for Δ7.

One obvious explanation for the failure of the K296R, G274P, and G279P mutants to show a dominant-negative phenotype would be if these mutant proteins were expressed at lower levels than the Δ6, Δ3, Δ7, and ΔK proteins. To address this possibility, we compared the relative levels of the different

mutant proteins by immunoblot analysis. In accord with the results shown in Fig. 3, all of the mutant kinases were produced at higher levels than the wild-type enzyme when expressed from the pEMBLyex4 vector as the only DAI construct in strain H1816 (compare lanes 2, 4, 6, 8, and 10 in Fig. 6B and lanes 2, 4, 6, 8, and 10 in Fig. 6C). Importantly, the three point mutants were produced at levels very similar to those of the other four inactive proteins containing deletions in the kinase domain. When the mutant and wild-type enzymes were coexpressed in strain RY1-1, we generally found that the mutant proteins accumulated to somewhat lower levels than they did in strain H1816. The down-regulation of the mutant enzymes in strain RY1-1 can be accounted for by noting that none of the mutant kinases completely inactivates the wild-type enzyme; hence, wild-type DAI can still partially down-regulate synthesis of all the mutant proteins. Similar to what was seen in H1816, the levels of proteins containing point mutations were very similar to those of the other inactive kinases in strain RY1-1 (compare lanes 3, 5, 7, and 9 in Fig. 6B and lanes 1, 3, 5, and 7 in Fig. 6C). Consequently, the inability of the point mutants to interfere with the function of wild-type DAI cannot be explained by a failure to accumulate to the same levels as the four dominant-negative proteins. We also verified that the levels of the wild-type enzyme were very similar in all of the doubly transformed strains by assaying its autokinase and eIF-2α kinase activities in immune complexes isolated from the RY1-1 transformants, using the methods described for Fig. 5 (data not shown). These results suggest that dominant interference with wild-type DAI in yeast cells depends on disrupting a particular interaction between mutant and wild-type enzymes associated in a heterodimer, rather than resulting from sequestration of dsRNA activators by catalytically inactive proteins.

It is important to note that the mutant constructs with deletions in the kinase domain were not completely dominant to wild-type DAI. Although they decreased or reversed the growth inhibition caused by high-level expression of wild-type DAI on galactose medium, they did not prevent the induction of *GCN4* translation and the attendant 3-AT resistance when mutant and wild-type enzymes were expressed at low levels on SD medium. Furthermore, they did not appear to reduce negative autoregulation of kinase expression by the wild-type enzyme (Fig. 6B and C). Thus, in accord with previous results (2, 35), higher levels of eIF-2 phosphorylation are required for the general inhibition of protein synthesis and cell growth versus gene-specific translational control of DAI and *GCN4* expression. The incomplete dominance that we observed for the dominant-negative proteins could indicate that the heterodimers that they form with wild-type DAI are only partially impaired for kinase function. Alternatively, the residual DAI activity may arise primarily from the continued formation of wild-type homodimers in the strains coexpressing mutant and wild-type proteins.

Genetic complementation between DAI mutants in vivo. The idea that DAI functions as a dimer in yeast cells is supported by our finding that two mutant alleles in the low-activity class containing different mutations in the dsRNA-binding domain are able to complement one another and produce higher levels of DAI kinase function in vivo. To detect complementation between mutations in the first and second repeats of the dsRNA-binding motif, we inserted the Δ2 mutant construct into a different high-copy-number plasmid and introduced it into the *gcn2*Δ strain H1894. The resulting transformant grew slowly on galactose medium (Fig. 7A), in accord with the results obtained for this allele when it was expressed from pEMBLyex4 (Table 1). This strain was then cotransformed with a number of DAI alleles containing mutations in the first repeat borne on the pEMBLyex4 plasmids described above. Analysis of the doubly transformed strains revealed that the strain containing both Δ1 and Δ2 could not grow on galactose medium (Fig. 7B), indicating a restoration of DAI function. In contrast, strains bearing Δ2 combined with either LS16 or LS9, both of which contain very deleterious point mutations in the first repeat, or combined with a second copy of Δ2 all grew well on galactose medium. Recall that Δ2 is slightly more functional than Δ1 in singly transformed strains (Fig. 7A; Table 1) and that Δ1 and Δ2 appear to be expressed at very similar levels (Fig. 3). The fact that coexpressing Δ1 and Δ2 yields significantly increased kinase function, whereas simply increasing the dosage of Δ2 does not, strongly suggests that the Δ1 and Δ2 proteins functionally complemented one another.

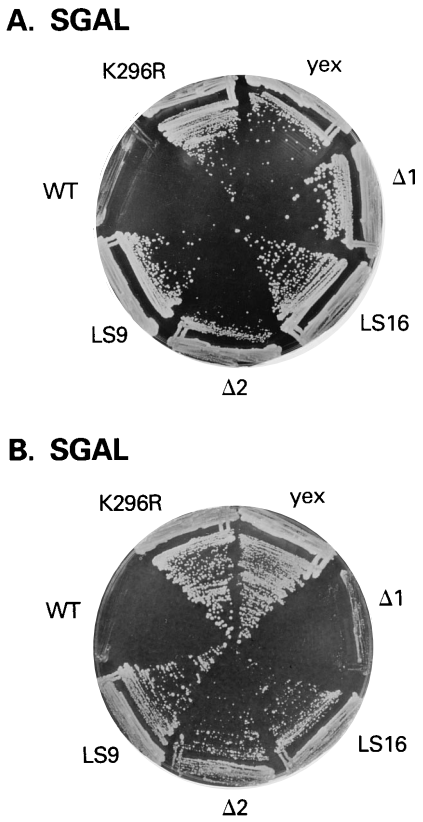

FIG. 7. Growth rate analysis showing functional complementation of DAI mutants Δ1 and Δ2. (A) Transformants of strain H1894 expressing the indicated DAI alleles were streaked on SGAL medium and incubated for 5 to 7 days at 30°C. WT, wild type. (B) Strain H1894 transformed with p1549, containing the DAI Δ2 allele on a high-copy-number vector, was additionally transformed with the indicated DAI alleles on pEMBLyex4 plasmids. Strains were streaked on SGAL (10% galactose, 2% raffinose) and incubated for 5 to 7 days at 30°C.

DISCUSSION

Efficient activation of DAI in vivo requires two copies of the dsRNA-binding motif and is correlated with dsRNA binding in vitro. We have exploited the fact that DAI can phosphorylate Ser-51 on eIF-2α in yeast cells to study the requirements for activation of DAI function in vivo. A large amount of biochemical data indicates that efficient binding of dsRNA by the N-terminal domain of DAI is dependent on both copies of the dsRNA-binding motif and that the first repeat plays a more important role than the second in this binding activity. Our results demonstrate that the structural requirements within the

repeated motifs for efficient dsRNA binding in vitro and for efficient kinase activation in yeast cells largely coincide. This concordance provides strong support for the widely held belief that activation of DAI in vivo is dependent on binding of dsRNA to the repeated motifs in the N-terminal domain of the protein.

The complete deletions of the first and second repeats (Δ1 and Δ2) and substitution of the first repeat with another copy of the second (construct 2:2) abrogated dsRNA binding in vitro and severely diminished kinase function in vivo. Substituting the second repeat with an additional copy of the first (1:1) left dsRNA binding in vitro largely intact and, if anything, led to slightly higher levels of kinase function in vivo (Table 1). These results indicate that (i) two copies of the repeat are required for dsRNA binding and kinase activation in vivo and (ii) the first copy of the repeat can substitute for the second, whereas the second cannot substitute for the first, either in dsRNA binding or in kinase activation. The two repeats function independently to some extent because switching their order in the N terminus of the protein (construct 2:1) actually leads to dsRNA binding and in vivo kinase activation greater than that seen for the wild-type enzyme.

The only point mutations in the dsRNA-binding domains comparable to Δ1 and Δ2 for impairing kinase activation in vivo (LS9 and LS16) would be expected to disrupt the predicted basic α-helical region in the C-terminal one-third of the first repeat. This is the most highly conserved portion of the dsRNA-binding motif, and the first repeat in DAI is a better match with the consensus sequence than is the second (38). As would be expected from the sequence conservation, the point mutations in LS9 and LS16 abolish dsRNA binding in vitro. Four of the five alleles in the group next most defective for in vivo kinase function, and which also show little or no dsRNA binding in vitro, contain substitutions at three different positions in the first repeat (LS13, LS4, LS2, and LS15). The fifth allele in this group contains the proline substitution that is expected to disrupt the predicted α-helical segment in the second repeat (LS19), underscoring the importance of this segment of the repeat in kinase activation. It is noteworthy that LS19 shows higher levels of kinase activity in vivo and dsRNA binding in vitro than were seen for LS16, because these two alleles contain proline substitutions at the equivalent alanine residues in the second and first repeats, respectively. Similarly, substitution of the conserved Phe-41 with alanine in the first repeat (LS15) leads to a significant reduction in kinase activity and dsRNA binding, whereas replacement of the equivalent Phe-131 in the second repeat (LS18) has little or no effect on either function. These results add strong support to the idea that the first repeat is more important than the second for kinase activation in vivo and for dsRNA binding in vitro.

This simple relationship does not hold, however, for LS17 and LS14. In this case, the mutation in the second repeat (LS17) has an equivalent (or perhaps more severe) effect on in vivo kinase function compared with the corresponding mutation in the first repeat (LS14), whereas LS14 is more defective than LS17 for dsRNA binding. On the other hand, the clustered substitutions in the N-terminal portion of the first repeat made by the LS2 mutation are more severe than those made at adjacent positions in the second repeat by the LS6 and LS12 mutations. The members of this last group of mutations affecting conserved residues in the N-terminal portions of the two repeats deserve additional comment because they have differential effects on dsRNA binding in vitro and kinase activation in vivo. For example, LS6 was more defective than LS17 for dsRNA binding in vitro, but by all criteria, LS6 confers higher levels of kinase activity in vivo. It is possible that mutations in the N-terminal portions of the two binding motifs affect a mechanism for coupling dsRNA binding to kinase activation as well as diminishing the efficiency of dsRNA binding. For all of the mutations besides LS6 and LS17, however, there is a good correspondence between the ordering of the mutations by the efficiency of dsRNA binding in vitro and by the level of kinase activity conferred in yeast cells. This finding indicates that the deleterious effects on kinase activation for the majority of the mutations that we studied can be attributed primarily to decreased binding of dsRNA.

Dimerization of DAI and the mechanism of dominant-negative mutations. Previous studies in mammalian cells indicated that the catalytically defective DAI alleles K296R and Δ6 can interfere with the function of wild-type DAI. Analysis of the K296R protein in cell-free translation systems indicated that the mutant protein reduced the activity of endogenous DAI in the extract by sequestering dsRNA activators (36), and this same mechanism was invoked to explain how overexpression of only the dsRNA-binding domain interferes with coexpressed wild-type enzyme in yeast cells (3). Inconsistent with this mechanism, however, we found that overexpression of the K296R, G274P, and G279P mutant proteins had no detectable dominant-negative phenotype, even though they were expressed at levels very similar to those seen for the ΔK, Δ6, Δ7, and Δ3 proteins that were dominant negative. Because binding of dsRNA appears to be completely independent of the kinase domain (7, 11, 28, 34), all seven of the catalytically inactive proteins that we examined are expected to bind dsRNA with wild-type efficiency and thus should be equally effective in sequestering dsRNA activators. Indeed, the K296R protein had this property when added in large amounts to a cell-free translation system (36).

An alternative explanation for the dominant-negative phenotypes that we observed would be that the Δ6, Δ3, Δ7, and ΔK proteins interfere with the function of wild-type DAI by forming defective heterodimers. This mechanism was suggested previously to account for the dominant-negative effect of the Δ6 allele in mammalian cells (19). In this model, mutant DAI proteins with defects in the dsRNA-binding motifs (which were all recessive in *S. cerevisiae*) would not interfere with the function of wild-type DAI, either because they cannot dimerize or because they form active heterodimers with the wild-type enzyme. Our recent finding that a Δ1-Δ6 double mutation shows a dominant-negative phenotype that is much less severe than that shown by the Δ6 single mutation (data not shown) suggests that deletion of the first dsRNA-binding repeat reduces but does not abolish dimer formation. The relative inactivity of heterodimers formed between wild-type DAI and the dominant-negative allele Δ6, Δ7, Δ3, or ΔK could relate to the fact that these proteins all have substantial alterations in the kinase domain. The ΔK protein lacks the kinase domain entirely, whereas the Δ7 and Δ6 alleles contain six-residue deletions in kinase subdomains II and V, respectively. The Δ3 mutation removes resi-dues immediately upstream of the first conserved glycine in kinase subdomain I, which are conserved among many different protein kinases (12) and which comprise the β-1 strand in the catalytic core of cyclic AMP-dependent protein kinase (18).

We propose that large structural alterations in the Δ6, Δ7, Δ3, and ΔK proteins decrease an interaction between the two kinase domains that is essential for the eIF-2α kinase activity of DAI (Fig. 8). This interaction could be required for activation of the autokinase function of the enzyme by dsRNA; alternatively, it could be needed for substrate binding or catalysis in the phosphorylation of eIF-2α. Presumably, the single

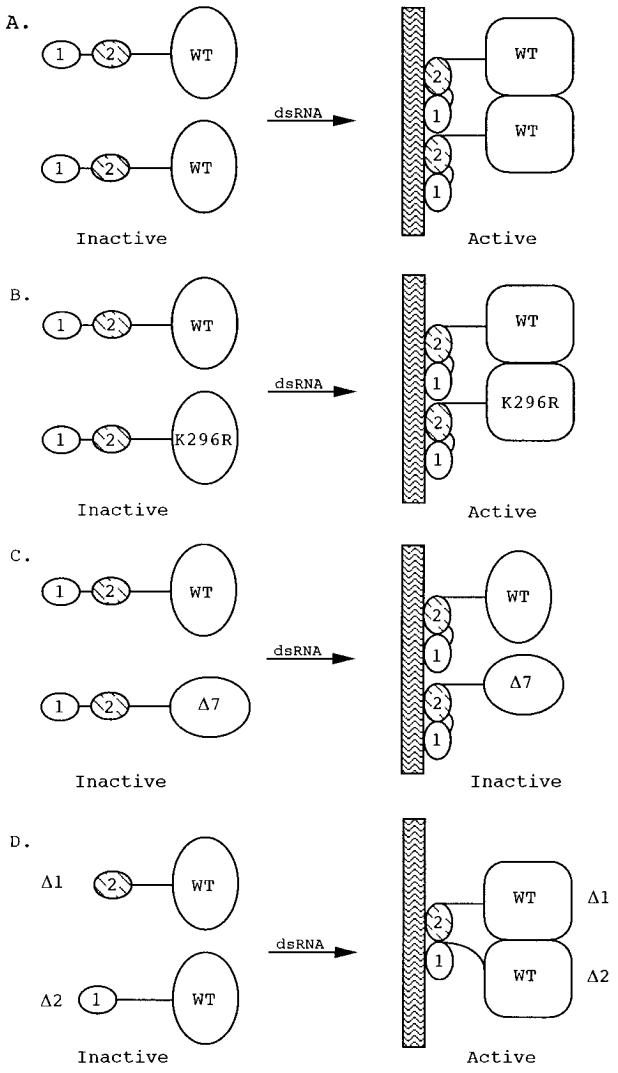

FIG. 8. Model for the role of dimer formation in the activation of DAI kinase function. DAI is shown schematically, with the two dsRNA-binding motifs labeled 1 and 2 and the protein kinase domain labeled according to whether it is wild-type (WT) or mutant (K296 or Δ7). The kinase domain is drawn as an oval when latent and a square when its eIF-2α kinase function is activated; dsRNA is shown as a stippled bar. (A) Upon binding dsRNA, two latent wild-type molecules of DAI form a dimer in which the eIF-2α kinase function of the enzyme is activated. This involves a specific interaction between the catalytic domains of the two protomers. The interaction between protomers could be required to stimulate autokinase activity, which in turn would activate the eIF-2α kinase function, or it could be required specifically to stimulate a unique aspect of eIF-2α kinase activity (substrate binding or catalysis). (B) A wild-type molecule can form an active heterodimer with a catalytically inactive protein bearing a single amino acid substitution in kinase subdomain II. The point mutation does not disrupt the structure of the mutant kinase domain sufficiently to prevent the interaction between protomers needed to activate the wild-type partner of the dimer. It is assumed that having only one functional protomer in the DAI dimer is sufficient to achieve essentially wild-type levels of eIF-2α phosphorylation in the cell. (C) A heterodimer containing a wild-type protomer and a catalytically inactive promoter with a deletion in kinase subdomain II is defective for kinase function, because the deletion in the mutant protein leads to a structural deformation that prevents proper interaction between the kinase domains of the two protomers. (D) The Δ1 and Δ2 protomers can form a heterodimer that is active for eIF-2α phosphorylation. This involves a juxtaposition of dsRNA-binding motif 2 from the Δ1 protomer with motif 1 from the Δ2 protomer in a way that permits the two kinase domains to interact productively.

amino acid substitutions in the K296R, G274P, and G279P proteins would not destroy the structural features necessary for the productive interaction between catalytic domains in the DAI dimer postulated in our model (Fig. 8). The K296R mutation is a conservative substitution that is predicted to alter the β-3 strand of the kinase domain. The G274P and G279P mutations are expected to insert prolines in the turn between the β-1 and β-2 strands (Gly-274) and in the β-2 strand (Gly-279), respectively (18). Although these proline substitutions would probably alter the flexibility of the β strands in which they reside, they would not be expected to alter the arrangement of neighboring structural elements as drastically as would Δ3 and Δ7, which delete most of the β-1 and β-3 strands, respectively.

In the immunoblot analysis of catalytically inactive DAI proteins that were coexpressed with wild-type DAI, we frequently observed that the mobility of the mutant protein was decreased relative to its mobility when it was expressed as the only form of DAI in the cell (Fig. 6B and C). This decreased mobility probably reflects intermolecular phosphorylation by the wild-type enzyme. This finding could be interpreted to indicate that the heterodimers formed between the mutants with deletions in the catalytic domain and wild-type DAI are fully active, rather than being defective as suggested above. It should be noted, however, that the phosphorylation of mutant enzymes observed in these experiments could be catalyzed by wild-type homodimers that are present in the same cells with the defective heterodimers. Alternatively, the heterodimers may not be completely defective and may become activated only after the cells are broken, analogous to the activation of Δ1 and Δ2 that occurs in vitro (Fig. 5). Another possibility is that only a subset of sites required for activation are being phosphorylated in the defective heterodimers or that the heterodimers are defective for eIF-2α phosphorylation but not for autophosphorylation.

Our finding that high-level expression of the K296R protein does not interfere with wild-type DAI seems to be at odds with two previous studies in which the K296R protein showed a dominant-negative effect on the wild-type enzyme. Barber et al. (2) demonstrated that expression of a reporter gene was greater in COS-1 cells transfected with the K296R DAI mutant allele than in COS-1 cells transfected with vector alone. They proposed that the K296R mutant formed inactive heterodimers with the endogenous enzyme and thereby stimulated translation of the reporter gene mRNA (2). To account for the observation that K296R is dominant-negative in COS-1 cells but not in our yeast system, it could be proposed that levels of dsRNA activator are much lower in COS-1 cells than in yeast cells. Because the endogenous monkey DAI kinase was not induced with interferon and presumably was present at low levels relative to the K296R mutant, most of the K296R protein would have existed in the form of inactive homodimers. If dsRNA activators are limiting in the COS-1 cells, as we suggest, then most of the dsRNA would be bound to these inactive K296R homodimers and be unavailable to activate the wild-type enzyme, whether present as K296R/wild-type heterodimers or as wild-type homodimers. In fact, Meurs et al. showed that the K296R mutant did not have a dominant-negative effect on eIF-2α phosphorylation in encephalomyocarditis-infected NIH 3T3 cells, in which the levels of dsRNA activators should be much higher (32).

The K296R protein was also found to interfere with wild-type DAI in rabbit reticulocyte lysates (36). The fact that this interference could be overcome at elevated concentrations of dsRNA indicates that the K296R protein was sequestering dsRNA activators in the lysate rather than forming inactive heterodimers with the wild-type enzyme. Because the endog-

enous enzyme was not induced in these lysates, the K296R protein was probably present in great excess over wild-type DAI. Thus, these results are consistent with the idea that the K296R protein can interfere with wild-type DAI only when present in large excess over the wild-type enzyme and when dsRNA activators are limiting. We suggest that these conditions did not prevail in our experiments because of high levels of dsRNA in yeast cells and that the primary mechanism for dominant interference in *S. cerevisiae* involves the formation of inactive heterodimers. Our results may have important implications in determining whether a particular DAI allele will be dominant negative and lead to oncogenic transformation in a given mammalian cell type.

The idea that the dominant-negative phenotype of mutant DAI proteins involves formation of defective heterodimers is also consistent with our observation that the Δ1 and Δ2 mutants functionally complemented in yeast cells. Coexpressing Δ1 and Δ2 led to higher levels of DAI function than occurred when Δ2 expression alone was boosted or when Δ2 was coexpressed with other mutant kinases that are comparably impaired for activation in vivo. The specificity of the interaction between Δ1 and Δ2 suggests that heterodimers containing these two proteins are more active than either homodimers of Δ1 or Δ2 or heterodimers between Δ2 and the other point mutants that we tested for complementation of Δ2. To explain how Δ1 and Δ2 functionally complemented, we propose that the remaining intact copies of the repeated motifs in the Δ1 and Δ2 proteins can be properly juxtaposed in the heterodimer to reconstitute a functional dsRNA-binding domain (Fig. 8D). To account for the fact that two copies of the first repeat in a Δ2/Δ2 dimer cannot similarly reconstitute dsRNA binding, it could be proposed that physical interactions between repeats 1 and 2 stabilize the dsRNA-binding domain formed in the Δ1/Δ2 dimer and that these important interactions are missing with two copies of repeat 1. For the defective dimers formed between Δ2 and the LS9 and LS16 point mutants, we suggest that the mutated first repeat in the LS9 and LS16 proteins blocks the intact first repeat in Δ2 from interacting productively with the intact second repeat in LS9 and LS16. Finally, we found that Δ2 cannot be complemented by a Δ1-Δ6 double mutation (data not shown), indicating that complementation requires an interaction between two structurally intact kinase domains that are juxtaposed through a single dsRNA-binding domain (Fig. 8D).

Our genetic findings are most easily explained by proposing that the active form of DAI is a dimer and that the catalytic domains of the two promoters must interact with one another in a specific fashion to achieve activation of eIF-2α kinase function. These findings are consistent with the possibility that the principal role of dsRNA binding is to mediate dimer formation on a single molecule of dsRNA. Close juxtaposition of two DAI protomers on the same dsRNA molecule would promote intermolecular autophosphorylation and the close interaction between catalytic domains that we postulate to be important for activation of eIF-2α kinase function. As noted above, this hypothesis explains the second-order kinetics of activation (20) and the fact that both short dsRNAs and high concentrations of dsRNA inhibit rather than activate the enzyme (25). It is also in accord with our recent finding that deletion of the first repeat in the dsRNA-binding domain reduces the ability of the Δ6 protein to interfere with wild-type DAI function, implying that dsRNA binding is required for dimer formation. It may be that dsRNA binding also induces a conformational change in the kinase domain of each protomer that is needed for kinase activation, in addition to stimulating dimer formation per se (25, 26). Finally, our conclusion that the ΔK protein interferes with wild-type DAI by forming inactive heterodimers leads to the interesting inference that the region of DAI N terminal to the catalytic domain (retained in ΔK) is sufficient to mediate dimer formation in vivo. We anticipate that analyzing additional DAI mutants for dominant-negative phenotypes and for functional complementation in yeast cells will yield further insights into the mechanism of DAI activation by dsRNA and the role of protein dimerization in this process.

ACKNOWLEDGMENTS

We are grateful to Nahum Sonenberg for providing the Δ6 mutant allele, Bill Merrick for the purified rabbit eIF-2, Tom Dever and Graham Pavitt for comments on the manuscript, and Kathy Shoobridge for help in preparation of the manuscript.

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